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## Labeling Cells with Silver/Dendrimer Nanocomposites

Wojciech Lesniak<sup>1</sup>, Xiangyang Shi<sup>1</sup>, Anna Bielinska<sup>1</sup>, Katarzyna Janczak<sup>1</sup>, Kai Sun<sup>2</sup> James R. Baker Jr.<sup>1</sup>, and Lajos P. Balogh<sup>1,3,4</sup>

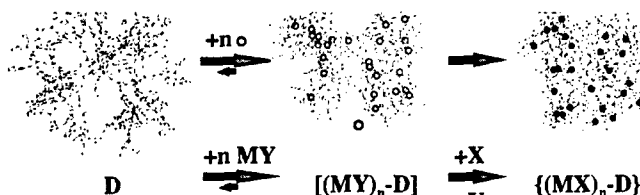
<sup>1</sup>Center for Biologic Nanotechnology, <sup>2</sup>Electron Microbeam Analysis Laboratory <sup>3</sup>Department of Biomedical Engineering, <sup>4</sup>Macromolecular Science and Engineering, University of Michigan, Ann Arbor, MI 48109

### Abstract

We have developed water-soluble, biocompatible, fluorescent, and photostable silver/dendrimer nanocomposites that have a potential to be used for *in vitro* cell labeling. A PAMAM\_E5.NH<sub>2</sub> dendrimer was used as a template to prepare first a silver-dendrimer complex in an aqueous solution at biologic pH=7.4. Conversion into nanocomposite was achieved by irradiating the solution of the  $\{(Ag^+)_{25}\text{-PAMAM\_E5.NH}_2\}$  complex by UV light to reduce the bound Ag<sup>+</sup> to zero-valent Ag<sup>0</sup> atoms, which were simultaneously trapped in the dendrimer network. Results indicate that the  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  silver/dendrimer nanocomposite forms positively charged single particles of 4-5 nm under the experimental conditions used. The dendrimer nanocomposite proved to be fluorescent. Toxicity testing of  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite revealed a behavior similar to the template dendrimer. Intracellular internalization of the silver nanocomposite and cell labeling capabilities was confirmed by confocal microscopy.

### Introduction

Nanostructured materials have gained increasing attention in the past decades due to their unique properties that provide outstanding opportunities for application in photonics, biology, and medicine. Dendrimer nanocomposites (DNC) [1-3] are nanosized inorganic/organic hybrid composite particles containing topologically trapped guest atoms/molecules/nanocrystals immobilized by dendritic polymer hosts of well-defined size, charge, and terminal functionality. Our goal here is to describe the synthesis, characterization and use of a particular class of silver nanocomposites that can be used as cell labels in *in vitro* experiments. Noble metal nanoparticles have been shown to demonstrate a strong, discrete emission quantum yield that is comparable to bright organic fluorophores, as they exhibit features useful in the biological imaging [4-5]. The properties of metal nanocomposites strongly depend on their size and shape [6-8]. Thus, control of nanoparticle morphology plays an essential role in their future applications. Nanocomposites that can be used in biological systems are required to be water soluble, biocompatible, and photostable. It is well documented that poly(amidoamine) (PAMAM) dendrimers hold great promise in this regard due to their highly regular, symmetrically branched structure with variable surface functionalities, which permits optimization for toxicity. PAMAMs can host inorganic ions or atoms/molecules by forming dendrimer complexes and nanocomposites [9]. Fabrication of metal/dendrimer nanocomposites requires two steps: (1) binding of metal ions to form complexes that act as precursors for consequent nanoclusters formation, and (2) transformation of resulting species to immobilize the preorganized metal ions within dendrimers, as depicted in scheme 1. Using the above principles, we have developed a novel approach for the fabrication of silver/dendrimer nanocomposites that allows to monitor of the chelation of Ag<sup>+</sup> in dendrimers, estimate their binding capacity and reduce metal ions at a desired pH.



**Scheme 1.** Preparation of dendrimer nanocomposites (DNC) by reactive encapsulation

It must also be pointed out that size distribution of nanocomposites depends on many factors such as: concentration of template, the metal/template molar ratio, pH, and temperature [10].

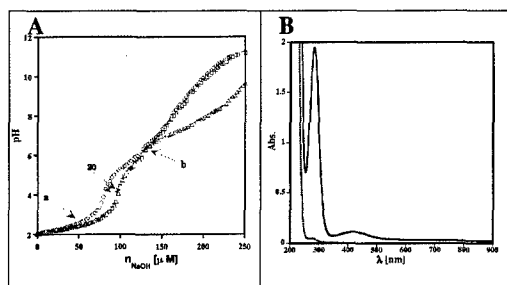
### Experimental

EDA-core PAMAM poly(amidoamine) dendrimer of generation 5 (PAMAM\_E5.NH<sub>2</sub>) in methanol solution (14.17wt%) was purchased from Dendritech (Midland, MI). Silver nitrate, and all the other chemicals and solvents were obtained from Aldrich and used as received. The dry form of PAMAM\_E5.NH<sub>2</sub> dendrimer was obtained by evaporating the methanol on a rotary evaporator and it was used in further studies without additional purification. Potentiometric titrations were performed manually, under nitrogen atmosphere, at room temperature, and over pH range of 2.5 - 12, using 0.1021 M NaOH standard solution. Concentration of PAMAM\_E5.NH<sub>2</sub> was set to  $3.7 \times 10^{-5}$  M, and the Ag<sup>+</sup>/dendrimer (i.e., the silver to dendrimer molar ratios) were set to 15:1, 30, 45:1. Details for {(Ag<sup>0</sup>)<sub>25</sub>-PAMAM\_E5.NH<sub>2</sub>} fabrication are included in the Results and Discussion section. The aqueous solutions of {(Ag<sup>0</sup>)<sub>25</sub>-PAMAM\_E5.NH<sub>2</sub>} nanocomposite was analyzed using a Perkin-Elmer Lambda 20 UV-Vis spectrophotometer, Fluoromax 3 spectrofluorometer, and a NICOMP 380/ZLS Zeta Potential Analyzer. HR-TEM, SAED and EDS data were acquired using a Jeol 2010F electron microscope operating at 200 kV. Samples were prepared by mounting a drop of aqueous solutions of nanoparticles on carbon-coated copper grids and allowed to dry in air. Cytotoxicity of dendrimer and its silver nanocomposites was measured using KB cells and XTT colorimetric assay of cellular viability (Roche Diagnostic, cat no: 1465 015), according to the manufacturer's instructions. Intracellular uptake of {(Ag<sup>0</sup>)<sub>25</sub>-PAMAM\_E5.NH<sub>2</sub>} nanoparticles by Rat2 cells was examined by Zeiss LSM 510 confocal microscope equipped with UV laser operating at 364 nm and an emission filter that transmits light between 465 and 485 nm.

### Results and Discussion

Figure 1A presents potentiometric titration curves of Ag<sup>+</sup>-PAMAM\_E5.NH<sub>2</sub> systems mixed at 15:1, 30:1 and 45:1 metal to dendrimer molar ratios. In the samples containing Ag<sup>+</sup>:PAMAM\_E5.NH<sub>2</sub> at 15:1 and 30:1 molar ratios, no precipitation was observed over the investigated pH range, indicating that 15 and 30 mol equivalent of silver ions was bound by dendrimer. Complexation of Ag<sup>+</sup> to the PAMAM\_E5.NH<sub>2</sub> dendrimer induces deprotonation of the binding sites, which is reflected in the different shape of the curves related to the titration of the dendrimer in the absence (circles) and presence (squares) of silver ions. Comparison of both titration curves reveals that binding of Ag<sup>+</sup> ions to PAMAM\_E5.NH<sub>2</sub> occurs in the pH range of 2.8 - 7 (marked in the graph as range *a* - *b*). As indicated in the graph, a 30 mol equivalent of H<sup>+</sup> ions was released during this process. Above pH 7 both curves are similar, suggesting that the resulting species are stable enough to withstand hydrolysis in the basic environment. On the

contrary, in the  $\text{Ag}^+:\text{PAMAM\_E5.NH}_2 = 45:1$  system, the precipitation of unbound  $\text{Ag}^+$  ions in the form of silver hydroxide was observed. The hydrolysis of unbound  $\text{Ag}^+$  starts at around  $\text{pH} = 7.5$ , which is evidently visible from the titration curve associated with this sample (triangles). Based on potentiometric data the synthesis of  $\{(\text{Ag}^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanoparticles was designed. Briefly, the  $\{(\text{Ag}^+)_{25}\text{-PAMAM\_E5.NH}_2\}$  precursor complex was obtained by mixing aqueous solutions of  $\text{Ag}^+$  ions and dendrimer template at 25:1 molar ratio and  $\text{pH} 2.5$ , which was subsequently increased to  $\text{pH} 7.4$  by titrating the system with  $\text{NaOH}$  solution to get the final dendrimer concentration equal to  $1\text{mg/mL}$ . To avoid additional chemical contamination, the dendrimer complex were reduced by UV irradiation. After irradiation, the originally colorless aqueous solution of  $\{(\text{Ag}^+)_{25}\text{-PAMAM\_E5.NH}_2\}$  species, became transparent yellow-brown, which confirmed the reduction of  $\text{Ag}^+$  to  $\text{Ag}^0$  and the formation of  $\{(\text{Ag}^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite. The UV-Vis spectra of the UV light exposed  $\text{PAMAM\_E5.NH}_2$ , dendrimer template and corresponding silver DNC are presented in figure 1B.



**Figure 1.** A - Potentiometric titration curves of  $\text{PAMAM\_E5.NH}_2$  (circles)  $\text{Ag}^+:\text{PAMAM\_E5.NH}_2$  30:1 (squares) and  $\text{Ag}^+:\text{PAMAM\_E5.NH}_2$  45:1 systems (triangles). B - UV-vis spectra of UV light exposed  $\text{PAMAM\_E5.NH}_2$  dendrimer (dotted line) and  $\{(\text{Ag}^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanoparticles (solid line), recorded for aqueous samples at  $\text{pH} 7.4$ .

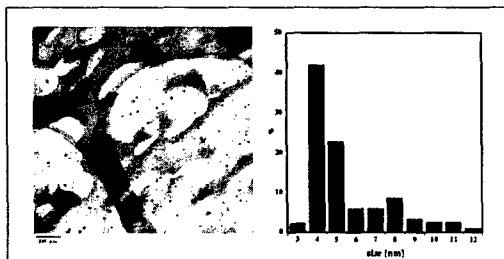
Solutions of  $\{(\text{Ag}^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite exhibits two absorbance peaks at 285 and 416 nm. The signal at 416 nm can be assigned as a plasmon peak that results from dipole resonances of aggregated silver nanoparticles. However, absorbance peaks at 285 nm may be related either to single particles or to partially oxidized dendrimer molecules that can form during photolytic reduction of  $\text{Ag}^+$  cations [11]. Silver complexes are known to be efficient oxygen transfer catalysts, which is in agreement with a recent report showing increase of this band upon oxidation of PAMAM dendrimers [11].  $\{(\text{Ag}^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanoparticles were further analyzed by means of dynamic light scattering and zeta potential measurements.

**Table 1** Summary of dynamic light scattering and average zeta potential data on  $\{(\text{Ag}^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite, obtained for a  $1\text{ mg/mL}$  aqueous sample at  $\text{pH}=7.4$ .

Number Weighting Mean Diam [nm]	Volume Weighting Mean Diam [nm]	IntensityWeighting Mean Diam [nm]	Zeta Potential
10.8 (99.6%) $\pm 0.8$	11.1 (83.7 %) $\pm 1$	11.3 (4%) $\pm 1$	+19.45
37.8 (0.4%) $\pm 2.3$	38.7 (15.5%) $\pm 3.3$	40.5 (29.9%) $\pm 3.8$	
	149 (0.8%) $\pm 18.6$	154.6 (66.1%) $\pm 17.5$	

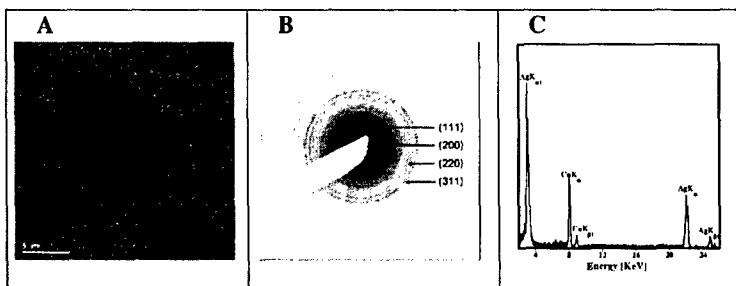
The  $\{(\text{Ag}^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite particles display relatively narrow size distribution with nanoparticles of hydrodynamic radius, for the most part, small particles and small fraction of higher order of aggregates that exhibit strong light scattering intensity. The particles are positively charged due to the fact that the terminal amino groups of the dendrimer

are still partially protonated at pH 7.4, which also indicates that terminal template functionality determines average charge of nanocomposite. Figure 3 shows a TEM image and related size distribution histograms obtained for the  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanoparticles based on evaluation of the images.



**Figure 3.** TEM image and related size distribution profiles of  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanoparticles was recorded at 92000x magnification. Size distribution histogram was obtained manually using the J-Image computer software.

The histogram indicates that the  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite is predominantly composed of single particles of diameter 4-5 nm. There is a difference between size distribution obtained from DLS and TEM imaging that results from the different underlying principles of the applied methods. First of all, preparation of the samples for TEM required dilution of the solution of  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  by a factor of 2, which may influence the aggregation degree in the system. DLS measurements are collected in solution, whereas for TEM, dry samples are used that may not reflect the exact particle distribution present in solution. Diameters measured from TEM images are only related to the location of the metallic domains in the nanocomposites without observing the organic host of the metal domains, while DLS measurements take into account the whole composite nanoparticle. Finally, due to the resolution threshold of the used particle-sizer ( $>5$  nm), particles with hydrodynamic radius of 11 nm and smaller could be considered as the same fraction close to the lower limit. Figure 4A presents an HRTEM image taken from an arbitrarily selected  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}_n$  aggregate particle suggesting that in this case a crystalline domain is formed. EDS spectroscopy confirmed that this is Ag domain. The lattice images obtained for the large  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite aggregates indicate that these particles contain either single crystalline or polycrystalline  $Ag^0$ . Figure 4B represent a typical SAED pattern crystalline of Ag domain collected from a number of particles.



**Figure 4.** HRTEM image of an aggregate particle; B: selected area electron diffraction (SAED) image, and C: EDS spectrum of the  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite.

Silver/dendrimer nanocomposites have been reported to be fluorescent [4-5]. Figure 5 shows the fluorescent spectra of the aqueous solution of  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  that contains fluorescent species that could be excited 340 nm, revealing emissions at 370 and 480 nm. Fluorescence may originate either from the oxidative byproducts, or from the nanosized silver domains (single or aggregated) and may be influenced by the surface aggregation states as well.

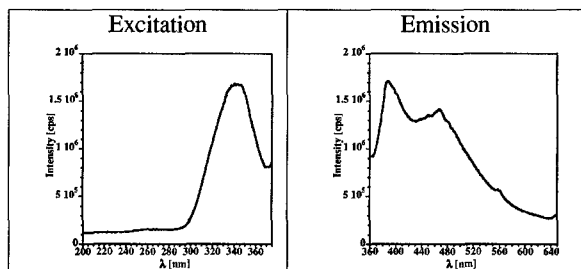
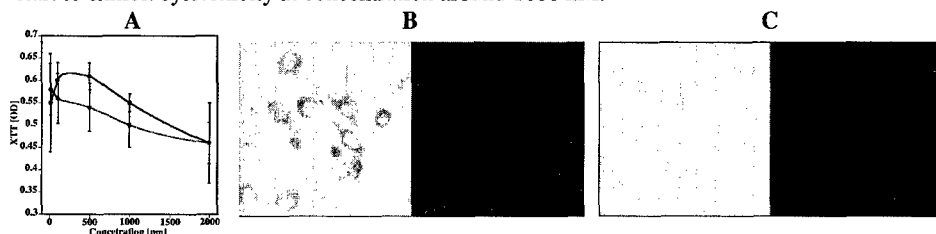


Figure 5. Excitation and emission spectra of the  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite, recorded in aqueous samples at pH =4.

Prompted by the observed fluorescence properties of  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanoparticles we further studied this material in terms of its potential application as a biomarker. As the first step, the cytotoxicity of the PAMAM\_E5.NH<sub>2</sub> dendrimer template and its related silver DNC was examined in KB cell culture using XTT colorimetric assay of cellular viability (Figure 5A). For PAMAM\_E5.NH<sub>2</sub> and  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  similar profiles were obtained, suggesting that dendrimer template determines the toxicity of nanocomposite. Both materials start to exhibit cytotoxicity at concentration around 1000 nM.



**Figure 5.** A: Toxicity evaluation of PAMAM-E5-NH<sub>2</sub> dendrimer and (dotted line)  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  (solid line) nanocomposite. KB cells were incubated with nanocomposites at concentration range of 1 and 2000 nM, for 2h at 37 °C in PBS buffer (pH 7.4). B: Inverted confocal images of Rat2 cells incubated with  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite at concentration of 500 nM, and C: control cells, recorded in the fluorescence (left panels) and DIC (right panel). Scanning was performed using Zeiss LSM 510 equipped with a UV laser operating at 364 nm and an emission filter that transmits light between 465 and 485 nm.

The cellular uptake and the observability of  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  DNC as a cell label was studied by means of confocal microscopy (Fig. 5 B and C). Both sets of images were collected under the same experimental conditions. Values of PMT and gain power were set to low to decrease the autofluorescence of cells. One can clearly see that cells incubated with  $\{(Ag^0)_{25}\text{-PAMAM\_E5.NH}_2\}$  nanocomposite exhibit higher intracellular fluorescence, indicating that (a) the nanoparticles are internalized within the cells and are visibly located in the

cytoplasm, and (b) the fluorescence is not quenched. All experiments were reproducible and intracellular enhancement of fluorescence was observed for the cells of each single experiment.

### Conclusion

We have demonstrated that potentiometric titration is a right tool to control metal binding by PAMAM dendrimers and to estimate binding capacity of these polymers at given pH values. Irradiation of the aqueous silver ion/dendrimer complex solutions (at pH=7.4) leads to the formation of fluorescent, biocompatible and highly photostable {Ag} dendrimer nanocomposite particles that may be used for cell labeling.

### Acknowledgement

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